

US\$N 10/500,943

**IN THE SPECIFICATION:**

The following is a marked-up version the Specification pursuant to revised 37 C.F.R. §1.121, with instructions and markings showing changes made herein to the Specification as filed. Underlining denotes added text while brackets denote deleted text.

On page 2, in the paragraph beginning at line 14, please enter the following amendments:

In another aspect of the present invention, a protease variant of a precursor protease is contemplated herein, the variant comprising one or more modifications at a charged amino acid residue position, the variant being characterized by having the same net electrostatic charge or isoelectric point as the precursor protease. The charged amino acids can be aspartic acid, glutamic acid, histidine, lysine, tyrosine and arginine. The residue positions can be those equivalent to positions 5, 7, 23, 26, 28-31, 34, 47, 63, 65, 66, 69, 70, 73, 82 - 85, 88, 90, 92, 93, 105, 113, 125, 138, 139, 148-151, 176, 178, 179, 193, 196, 200, 201, 202, 207, 219, 220, 223, 229, 233, 250, 266, 267 and 273 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3) are identified herein. The residue positions can also be those equivalent to positions 27, 39, 41, 45, 67, 94, 136, 170, 181, 247, 251 and/or 271 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3). It is a further aspect to provide DNA sequences encoding such protease variants, as well as expression vectors containing such variant DNA sequences.

On page 2, in the paragraph beginning at line 28 and continuing on page 2 to line 7, please enter the following amendments:

A protease variant of a precursor protease, said variant comprising one or more modifications at a charged amino acid residue position, said variant being characterized by having the same net electrostatic charge as said precursor protease. The protease variant of claim 1, wherein said charged amino acid is selected from the group consisting of aspartic acid, glutamic acid, lysine and arginine. The protease variant comprises an amino acid sequence having a substitution at one or more residue positions equivalent to residue positions selected from the group consisting of 27, 45, 170, 181, 251 and 271 of *Bacillus amyloliquefaciens* subtilisin as set forth in (SEQ ID NO:3) ~~SEQ ID NO:2~~. The protease variant comprising a substitution at one or more positions corresponding to 27, 45, 170, 181, 251 and 271 is a substitution selected from K27T, R45N, R170S, D181N, K251G and E271T.

On page 3, in the paragraph beginning at line 8, please enter the following amendments:

USSN 10/500,943

The protease variant may further comprise an additional substitution at one or more positions corresponding to 1, 14, 49, 61, 87, 100, 102, 118, 128, 204 and 258 of *Bacillus amyloliquefaciens* subtilisin as set forth in (SEQ ID NO:3) ~~SEQ ID NO:2~~. Variants can be selected from the combinations of R45N-G118E-E271R, R45N-P14R, R45N-N204R, D181N-G118D, R45N-G258R, R170S-A1R, R170S-G61R, R170S-N204R, K251G-S87K, R170S-S216R, E271T-G100E, E271T-G102E, E271T-S128E, K27T-G100E, R170S-G100R, E271T-S49E and E271T-S128E.

On page 15, in the paragraph beginning at line 5, please enter the following amendments:

In another aspect, the variant proteases of the present invention have, relative to said precursor protease, the same number of positively-charged amino acid residue(s), both the identical amino acids as in the precursor protease and different amino acids having the same charge, and the same number of negatively-charged amino acid residue(s) as in the precursor protease; or either more or fewer positively-charged amino acid residue(s) and a corresponding more or fewer negatively-charged amino acid residue(s), such that the net electrostatic charge and/or the isoelectric point of the protease variant is the same as the precursor protease, while having modifications among the equivalent amino acid residues at any one or more of positions: 5, 7, 22, 23, 24, 26, 28-31, 34, 45, 47, 63, 65, 66, 69, 70, 73, 82 - 85, 88, 90, 92, 93, 97, 102, 105, 113, 125, 127, 138, 139, 148-151, 169, 170, 176, 178, 179, 193, 196, 200, 201, 202, 207, 219, 220, 223, 229, 233, 250, 266, 267 and 273 of *Bacillus amyloliquefaciens* (BPN') (SEQ ID NO:3). In one embodiment, modifications among the equivalent amino acid residues at one or more of positions 27, 45, 136, 170, 181, 247, 251 and/or 271 include the substitution of an uncharged residue for a charged residue position. These residue positions are of interest since these equivalent positions in *Bacillus lentus* wild type have charged amino acid residues at these positions. For example, the residue positions at 27, 38, 40, 44, 65, 92, 134, 164, 175, 241, 245, and/or 265 of *Bacillus lentus* subtilisin (SEQ ID NO:6) (~~SEQ ID NO:6~~) are equivalent, respectively, to 27, 39, 41, 45, 67, 94, 136, 170, 181, 247, 251 and/or 271 of *Bacillus amyloliquefacien* (SEQ ID NO:3) (~~SEQ ID No. 2~~).

On page 15, in the paragraph beginning at line 27 and continuing through page 17, at line 11, please enter the following amendments:

In another aspect, the variant proteases of the present invention have, relative to said precursor protease, the same number of positively-charged amino acid residue(s), both the identical amino acids as in the precursor protease and different amino acids having the same

USSN 10/500,943

charge, and the same number of negatively-charged amino acid residue(s) as in the precursor protease; or either more or fewer positively-charged amino acid residue(s) and a corresponding more or fewer negatively-charged amino acid residue(s), such that the net electrostatic charge and/or the isoelectric point of the protease variant is the same as the precursor protease, while having modifications among the equivalent amino acid residues at any one or more of positions: 27, 39, 41, 45, 67, 94, 136, 170, 181, 197, 247, 249, 251, and 271 of *Bacillus amyloliquefaciens* (BPN') (SEQ ID NO:3). Specific substitutions contemplated by the inventors include K27A, K27C, K27E, K27Q, K27G, K27H, K27I, K27L, K27M, K27F, K27P, K27S, K27T, K27W, K27Y, H39A, H39R, H39D, H39N, H39C, H39E, H39Q, H39G, H39H, H39I, H39L, H39K, H39M, H39F, H39P, H39T, H39W, H39Y, H39V, D41A, D41R, D41C, D41E, D41Q, D41G, D41H, D41I, D41L, D41K, D41M, D41F, D41P, D41S, D41T, D41W, D41Y, D41V, R45A, R45R, R45D, R45N, R45C, R45E, R45Q, R45G, R45H, R45I, R45L, R45K, R45M, R45F, R45P, R45S, R45T, R45W, R45Y, R45V, H67A, H67R, H67D, H67N, H67C, H67E, H67Q, H67G, H67H, H67I, H67L, H67K, H67M, H67F, H67P, H67S, H67T, H67W, H67Y, H67V, K94A, K94R, K94D, K94N, K94C, K94E, K94Q, K94G, K94H, K94I, K94L, K94K, K94M, K94F, K94P, K94S, K94T, K94W, K94Y, K94V, E136A, E136D, E136N, E136C, E136E, E136G, E136H, E136I, E136L, E136K, E136M, E136F, E136P, E136S, E136T, E136W, E136Y, E136V, R170A, R170R, R170D, R170N, R170C, R170E, R170Q, R170G, R170H, R170I, R170L, R170K, R170M, R170F, R170P, R170S, R170T, R170W, R170Y, R170V, D181A, D181R, D181D, D181N, D181C, D181E, D181Q, D181G, D181H, D181I, D181L, D181K, D181M, D181F, D181P, D181S, D181T, D181W, D181Y, D181V, D197A, D197R, D197D, D197N, D197C, D197E, D197Q, D197G, D197H, D197I, D197L, D197K, D197M, D197F, D197P, D197S, D197T, D197W, D197Y, D197V, R247A, R247R, R247D, R247N, R247C, R247E, R247Q, R247G, R247H, R247I, R247L, R247K, R247M, R247F, R247P, R247S, R247T, R247W, R247Y, R247V, H249A, H249R, H249D, H249N, H249C, H249E, H249Q, H249G, H249H, H249I, H249K, H249M, H249F, H249P, H249S, H249T, H249W, H249V, K251A, K251D, K251C, K251Q, K251G, K251H, K251I, K251L, K251K, K251M, K251F, K251P, K251S, K251T, K251W, K251Y, K251V, E271A, E271R, E271D, E271N, E271C, E271E, E271H, E271I, E271L, E271K, E271M, E271F, E271P, E271S, E271T, E271W, E271Y, and/or E271V of *Bacillus amyloliquefaciens* (SEQ ID NO:3). It was noted that an increase in the number of positive charged residues by substitution thereof may result in an increase in the efficacy of that particular variant in a particular wash environment, while a corresponding opposite charge change could result in increased efficacy in a different wash environment. For example, it is

USSN 10/500,943

anticipated that negative charge mutations provide beneficial characteristics in low ionic strength wash environments and that positive charge mutations provide beneficial characteristics in high ionic strength wash environments. It is anticipated that variants that encompass both a positive increase and a negative increase while maintaining the same net electrostatic charge or isoelectric point will result in a protease molecule that exhibits improved characteristics in both environments as compared to the performance of the precursor protease.

These substitutions are preferably made in *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* protease, for example *Bacillus amyloliquefaciens* and/or Subtilisin 309 (SEQ ID NO:6).

On page 17, in the paragraph beginning at line 15, please enter the following amendments:

One aspect of the present invention includes a protease variant further comprising at least one additional replaced amino acid at one or more residue positions equivalent to residue positions or selected from the group consisting of 1, 2 – 4, 6, 9-12, 14, 15, 17-20, 25, 27, 36-38, 40, 44, 49, 51, 52, 54-61, 68, 71, 75, 76, 87, 89, 91, 97, 100-102, 104, 108, 111, 112, 115, 117, 118, 120-123, 128, 129, 131, 133, 134, 136, 137, 140, 143-146, 159, 164, 165, 167, 170, 171, 173, 175, 180, 182-187, 191, 192, 194, 195, 204, 206, 209-212, 216, 218, 222, 224, 226 234-245, 252, 255, 257-263 265, 268, 269, and 274 of *B. amyloliquefaciens* subtilisin (SEQ ID NO:3). Specific substitutions contemplated by the inventors include those equivalent to : I122A, Y195E, M222A, M222S, Y167A, R170S, A194P, D36, N76D, H120D, G195E, and K235N of *Bacillus amyloliquefaciens* (SEQ ID NO:3) or *Bacillus lentus*, (SEQ ID NO:6) which variant is derived from a *Bacillus* subtilisin.

On page 17, in the paragraph beginning at line 27 and continuing to line 13 on page 18, please enter the following amendments:

Of particular interest are variants at these positions demonstrating increased wash performance with a charged amino acid substitution. Combination variants including these positions and those originally having a charged amino acid are of interest. Exemplary combinations contemplated by the inventors include K27T-G100E, R45N-A1R, R45N-P14R, R45N-G61R, R45N-S128R, R45N-N204R, R45N-S216R, R45N-G258R, R170S-A1R, R170S-P14R, R170S-S49R, R170S-G61R, R170S-G100R, R170S-S128R, R170S-N204R, R170S-S216R, R170S-G258R, D181N-G118D, D181N-G258D, K251G-S87K, E271T-S49E, E271T-T66E, E271T-G100E, E271T-G102E, E271T-S128E, R45N-G118E-E271R, S49R-G102E-

USSN 10/500,943

R170S-E271T, and P14R-R45N-R170S-G258R. Those skilled in the art will recognize the protease variants having these modifications can be made and are described in US Patents 5,741,694; 6,190,900; and 6,197,567, expressly incorporated by reference herein. In addition, these modifications can also be made using direct *Bacillus* transformation methods as described in Provisional Application Ser. No. 60/423,087 (filed November 1, 2002; Neelam Amin and Volker Schellenberger). In one embodiment, the modifications were performed using fusion PCR techniques (Teplyakov, AV, et al, Protein Eng., 1992 Jul 5(5):413-20). Provisional Application Serial Number [ ] 60/440,792, filed concurrently this date (Chris Leeftang, et al.)

On page 18, in the paragraph beginning at line 14, please enter the following amendments:

Still another aspect of the present invention includes a protease variant further comprising at least one additional replaced amino acid at one or more residue positions from the group consisting of 21, 22, 24, 32, 33, 36, 50, 64, 67, 77, 87, 94, 95, 96, 97, 104, 107, 110, 124, 123, 126, 127, 128, 129, 135, 152, 155, 157, 156, 166, 169, 170, 171, 172, 189, 197, 204, 213, 214, 215, 217, 222, or 274 of *Bacillus amyloliquefaciens* (SEQ ID NO:3). Specific residues contemplated by the inventors include: K27R, M50F, N76D, S101G, S103A, V104I, V104Y, I122A, N123S, M124L, G159D, Y217L, A232V, Q236H, Q245R, N248D, N252K, T274A, and M222S. Protease variants, recombinant DNA encoding mutants at these positions and/or methods for making these modifications are described in US patent Nos. RE 34,606; 5,972,682; 5,185,258; 5,310,675; 5,316,941; 5,801,038; 5,972,682; 5,955,340 and 5,700,676, expressly incorporated by reference herein.

On page 19, in the paragraph beginning at line 27, please enter the following amendments:

For example, in Fig. 3, the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* (SEQ ID NO:3), *Bacillus subtilis* (SEQ ID NO:4), *Bacillus licheniformis* (carlsbergensis) (SEQ ID NO:5) and *Bacillus lentus* (SEQ ID NO:6) are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. These conserved residues (as between BPN' and *B. lentus*) are identified in Fig. 2.

On page 33, in the paragraph beginning at line 29, and continuing to line 25 of page 34, please enter the following amendments:

USSN 10/500,943

A large number of protease variants can be produced and purified using methods well known in the art. Mutations can be made in *Bacillus amyloliquefaciens* (BPN') subtilisin or *Bacillus lentus* GG36 subtilisin. The variants can be selected from the following:

K27A, K27C, K27E, K27Q, K27G, K27H, K27I, K27L, K27M, K27F, K27P, K27S, K27T, K27W, K27Y, H39A, H39R, H39D, H39N, H39C, H39E, H39Q, H39G, H39I, H39L, H39K, H39M, H39F, H39P, H39T, H39W, H39Y, H39V, D41A, D41R, D41C, D41E, D41Q, D41G, D41H, D41I, D41L, D41K, D41M, D41F, D41P, D41S, D41T, D41W, D41Y, D41V, R45A, R45D, R45N, R45C, R45E, R45Q, R45G, R45H, R45I, R45L, R45K, R45M, R45F, R45P, R45S, R45T, R45W, R45Y, R45V, H67A, H67R, H67D, H67N, H67C, H67E, H67Q, H67G, H67I, H67L, H67K, H67M, H67F, H67P, H67S, H67T, H67W, H67Y, H67V, K94A, K94R, K94D, K94N, K94C, K94E, K94Q, K94G, K94H, K94I, K94L, K94M, K94F, K94P, K94S, K94T, K94W, K94Y, K94V, E136A, E136D, E136N, E136C, E136G, E136H, E136I, E136L, E136K, E136M, E136F, E136P, E136S, E136T, E136W, E136Y, E136V, R170A, R170D, R170N, R170C, R170E, R170Q, R170G, R170H, R170I, R170L, R170K, R170M, R170F, R170P, R170S, R170T, R170W, R170Y, R170V, D181A, D181R, D181N, D181C, D181E, D181Q, D181G, D181H, D181I, D181L, D181K, D181M, D181F, D181P, D181S, D181T, D181W, D181Y, D181V, D197A, D197R, D197N, D197C, D197E, D197Q, D197G, D197H, D197I, D197L, D197K, D197M, D197F, D197P, D197S, D197T, D197W, D197Y, D197V, R247A, R247D, R247N, R247C, R247E, R247Q, R247G, R247H, R247I, R247L, R247K, R247M, R247F, R247P, R247S, R247T, R247W, R247Y, R247V, H249A, H249R, H249D, H249N, H249C, H249E, H249Q, H249G, H249I, H249K, H249M, H249F, H249P, H249S, H249T, H249W, H249V, K251A, K251D, K251C, K251Q, K251G, K251H, K251I, K251L, K251M, K251F, K251P, K251S, K251T, K251W, K251Y, K251V, E271A, E271R, E271D, E271N, E271C, E271H, E271I, E271L, E271K, E271M, E271F, E271P, E271S, E271T, E271W, E271Y, and/or E271V of *Bacillus amyloliquefaciens* (SEQ ID NO:3).

On page 34, in the paragraph beginning at line 27, and continuing to line 2 of page 35, please enter the following amendments:

A large number of protease variants can be produced and purified using methods well known in the art. Mutations can be made in *Bacillus amyloliquefaciens* (BPN') subtilisin (SEQ ID NO:3) or *Bacillus lentus* GG36 subtilisin (SEQ ID NO:6). The variants can be made with insertions, deletions or substitutions at the amino acids equivalent to those at positions: 5, 7, 23, 26, 28-31, 34, 47, 63, 65, 66, 69, 70, 73, 82 - 85, 88, 90, 92, 93, 105, 113, 125, 138, 139, 148-

RECEIVED  
CENTRAL FAX CENTER  
SEP 25 2006

USSN 10/500,943

151, 176, 178, 179, 193, 196, 200, 201, 202, 207, 219, 220, 223, 229, 233, 250, 266, 267 and 273 of *Bacillus amyloliquefaciens* (BPN') (SEQ ID NO:3).

On page 37, in the paragraph beginning at line 6, please enter the following amendment:

Forward *Apal* primer:

GTGTGTGGGCCCATCAGTCTGACGACC (SEQ ID NO:7)

On page 37, in the paragraph beginning at line 9, please enter the following amendment:

Reverse *Apal* primer:

GTGTGTGGGCCCTATTCGGATATTGAG (SEQ ID NO:8)

**LIST OF CLAIMS, SHOWING THE STATUS OF EACH CLAIM**

Underlining denotes added text while strikethrough denotes deleted text.

**IN THE CLAIMS:**

1. (Cancelled)

2. (Cancelled)

3. (Currently Amended) ~~The protease variant of claim 1, A protease variant of a precursor protease, said variant comprising one or more modifications at a charged amino acid residue position, said variant being characterized by having the same net electrostatic charge as said precursor protease, wherein said variant comprises an amino acid sequence having a substitution at one or more the residue positions equivalent to residue position 170, and further comprising a substitution at one or more positions corresponding to 27, 45, 181, 251 and 271 wherein the substitution is K27T, R45N, R170S, D181N, K251G or E271T of *Bacillus amyloliquefaciens* subtilisin as set forth in SEQ ID NO:3 SEQ ID NO:2.~~ The protease variant of claim 1, A protease variant of a precursor protease, said variant comprising one or more modifications at a charged amino acid residue position, said variant being characterized by having the same net electrostatic charge as said precursor protease, wherein said variant comprises an amino acid sequence having a substitution at one or more the residue positions equivalent to residue position 170, and further comprising a substitution at one or more positions corresponding to 27, 45, 181, 251 and 271 wherein the substitution is K27T, R45N, R170S, D181N, K251G or E271T of *Bacillus amyloliquefaciens* subtilisin as set forth in SEQ ID NO:3 SEQ ID NO:2.

4. (Cancelled)

5. (Currently Amended) The protease variant of claim 3, further comprising an additional substitution at position corresponding to position 87, of *Bacillus amyloliquefaciens* subtilisin as set forth in SEQ ID NO:3 SEQ ID NO:2.

6. (Cancelled)

USSN 10/500,943

7. (Currently Amended) A DNA encoding a the protease variant of claim 3 ~~2~~.
8. (Original) An expression vector encoding the DNA of claim 7.
9. (Original) A host cell transformed with the expression vector of claim 8.
10. (Currently Amended) A cleaning composition comprising the protease variant of claim 3 ~~2~~.
11. (Cancelled)
12. (Cancelled)